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(54) Title: METHOD OF INHIBITING RESTENOSIS USING CALRETICULIN (57) Abstract This invention relates to calreticulin, segments and derivatives thereof and to therapeutic compositions containing such products for treating restenosis. It relates also to methods of producing the products by chemical synthesis or employing recombinant techniques. The invention is also concerned with the use of the products for treating patients to prevent atherosclerosis development as well as recurrent plaque growth. A method of treating a patient to inhibit restenosis comprises administering to such patient in an amount which is effective to inhibit restenosis a compound selected from the group consisting of calreticulin; the C-domain of calreticulin; a C-domain containing segment of calreticulin; and a polypeptide which contains from about 6 to 100 amino acid residues and is an addition, substitution or deletion analog of the C-domain of calreticulin having the same functional activity. In a specific embodiment, the polypeptide has the amino acid sequence KEEEEKKRKEEEEAEEDEEDKDDKEDEDEDEEDKDEEEEEE.		

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METHOD OF INHIBITING RESTENOSIS USING CALRETICULIN

BACKGROUND OF THE INVENTION

This invention relates to calreticulin, segments and derivatives thereof and to therapeutic compositions containing such products. It relates also to methods of producing the products by chemical synthesis or employing recombinant techniques. The invention is also concerned with the use of the products for treating patients to prevent atherosclerosis development as well as recurrent plaque growth, restenosis.

Alternative approaches to coronary bypass surgery have been developed. In the most frequently utilized non-operative procedures for the improvement of blood flow in patients with coronary artery disease, a catheter with an inflatable balloon at the distal end is inserted into the femoral artery or by brachial cutdown, and is positioned by fluoroscopic control at the appropriate coronary ostium. The process is known as percutaneous transluminal coronary angioplasty (PTCA). Other approaches to recanalization of narrowed, stenotic or occluded arteries include laser angioplasty, atherectomy and stent implantation. Collectively these non-operative procedures are termed non-interventional procedures.

The balloon at the distal end of the catheter has a predetermined maximum diameter. It is filled with a radio opaque dye to permit visualization. Alternatively, the balloon itself may be radio opaque. When the balloon is positioned in the stenosis it is inflated at pressures of from 2 to 16 atmospheres for from 15 secs to 5 minutes and then deflated. The inflation cycle may be repeated several times to achieve satisfactory results. Normally the luminal diameter of the stenotic vessel increases at least 20% as a result of the treatment.

Angioplasty is not limited to the coronary arteries. It has been employed for treatment of single, large atherosclerotic lesions of the renal, iliac, and even carotic and vetebral arteries. The effect of the expanded balloon is to literally blow open the stenotic zone. Disruption of the wall is marked, including fracture and dissection of the lesion, and extravasation of the plaque lipid and connective tissue content into the adjacent vessel wall.

The clinical results of angioplasty include endothelial denudation, vascular wall damage, and rupture of the internal and external elastic laminae. These injuries have been found to result, in many cases, in unregulated proliferation of arterial smooth muscle cells (SMC) with resulting recurrent plaque growth restenosis. A recent study by Levine et al (1) has shown that restenosis may be expected to occur in as many as 25-50% of patients that have undergone angioplasty or other interventional procedures. Often the only practical treatment for restenosis is to repeat the treatment. This may cause further damage to the cell wall and the need for subsequent repetition of the angioplasty procedure or potentially open heart surgery or coronary bypass.

A principal object of this invention is the avoidance of the need for multiple angioplasties by preventing restenosis and for primary prevention of plaque development.

Calreticulin is a 46kDa protein (40kDa in SDS-PAGE) with a high proportion of acidic amino acids (109 acidic vs 52 basic). It was discovered in skeletal muscle sarcoplasmic reticulum over

twenty years ago (2). The amino acid sequence is shown in the Figure. The molecule is highly conserved as has been shown with calretoculin isolated from several different species. It is a major calcium binding/storage protein of the lumen of the endoplasmic reticulum (ER) (5). This unusual protein has also been found outside of the ER in the nuclear envelope, and in the nucleus in some cells. Cytoplasmic and extracellular localization of calreticulin has also been proposed (6,7). Calreticulin modulates steroid-sensitive gene expression (8,9) interacts with the α -subunit of integrin (6) and affects adhesion properties of different cells (10). The synthesis of calreticulin is induced in the stimulated human and mouse T-cells (11) and the protein is localized to the lytic granules in cytolytic T lymphocytes (12) suggesting that calreticulin may play a role in killing of target cells.

The structure of calreticulin has been firmly established. Structural analysis of its amino acid sequence indicates that the protein can be divided into at least three distinct domains (3,5). The N-domain of calreticulin is predicted to form anti-parallel α -strands with a helix-turn-helix motif at the extreme

NH₂-terminal. In newly synthesized calreticulin, the NH₂-terminal is extended with a hydrophobic ER signal sequence (13). The amino acid sequence of the N-domain shows no homology with any other protein sequence in current data bases, and it is also the most conserved domain among all calreticulins so far cloned.

The next domain of calreticulin has been designated the P-domain. This region of the protein is proline-rich and contains three sequence repeats of 17 amino acids (PxxlxDPDAXKPEDWDE). The amino acid sequence of the P-domain is highly conserved between species, and interestingly, an amino acid sequence very similar to this domain of calreticulin has been found in calnexin, an ER membrane protein proposed to function as a chaperone. Functionally, the P-domain binds 1 mole of Ca²⁺ per mole of protein with a dissociation constant of 1 uM indicating that this domain contains the high affinity Ca²⁺ binding site of calreticulin (3). The region of the P-domain contains the three repeats and is predicted to form a helix-loop-helix motif, similar to that found in EF-hand Ca²⁺ binding sites (14).

The C-domain contains 37 acidic residues and binds Ca^{2+} with high capacity/low affinity. This region probably represents the Ca^{2+} storage site of the protein as it binds 25 moles of Ca^{2+} /mole of protein (3). One potential glycosylation site is found in the C-domain (residue 326), along with the ER retention signal sequence KDEL (15). In addition to this, the C-domain of calreticulin interacts in vitro with a set of ER/SR proteins (8) as well as with the blood clotting factors IX and X and prothrombins (16,17). The interactions of the C-domain with these blood clotting factors may be responsible for the recently observed antithrombotic activity of calreticulin.

The C-domain of calreticulin shares limited amino acid sequence similarity with calsequestrin, and also with other proteins resident in the ER (PDI, BiP, endoplasmin) (5,13). Interestingly, the greatest divergence of sequence identity between calreticulins occurs in the COOH-terminal region. In particular, the C-domain of calreticulin from O. volvulus is positively, rather than negatively, charged and also does not terminate with the KDEL ER retention signal (18).

As shown in the figure,, the N-domain of calreticulin is from amino acid residue 1 to residue 180. The P-domain is from residue 181 to residue 290 and the C-domain, from residue 291 to residue 401.

The complete calreticulin molecule has been expressed in E. coli, as have the N, P, C, N & P and P&C domains (3). These domains were expressed using standard, well known techniques.

For the preparation of the products of this invention, conventional molecular biology, microbiology and recombinant DNA techniques within the skill of the art and fully explained in the literature (19, 20, 21, 22, 23, 24) may be employed.

The products of the present invention may be prepared using cloning vectors containing genes encoding analogs and derivatives having the functional activity of the C-domain of calreticulin. The production and use of such products are within the scope of the present invention. In a specific embodiment, the derivative or analog is functionally active, i.e., capable of exhibiting one or more functional activities associated with the C-domain of

calreticulin. The derivative or analog could have decreased activity or increased stability under the conditions in which it may be employed.

Products useful in the practice of the invention can also be made by altering encoding nucleic acid sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Preferably, derivatives are made that have enhanced or increased functional activity relative to calreticulin or its domains.

Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as calreticulin, its domains and derivatives thereof may be used in the practice of the present invention. These include but are not limited to allelic genes, homologous genes from other species, and nucleotide sequences comprising all or portions of appropriate genes which are altered by the substitution of different codons that encode the same amino acid residue within the sequence, thus producing a silent change. Likewise, the products of the invention include, but are not limited to, those

containing, as a primary amino acid sequence, all or part of the amino acid sequence of calreticulin, its domains or segments thereof including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a conservative amino acid substitution. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (acidic) amino acids include aspartic acid and glutamic acid. Such alterations will not be expected to affect apparent molecular weight as determined by polyarylamide gel electrophoresis. For situations where one charged amino acid is exchanged with another (similarly or differently charged, or uncharged) amino acid, the changes in pI will be more readily

detectable in small proteins whose pI falls within the interval between the pKas of the groups involved. Such a change may be imperceptible for large proteins.

The genes encoding products of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned gene sequence can be modified by any of numerous strategies known in the art (19). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated in vitro. In the production of the gene encoding a desired product, care should be taken to ensure that the modified gene remains within the same translational reading frame as the product gene, uninterrupted by translational stop signals, in the gene region where the desired activity is encoded.

Additionally, the nucleic acid sequence can be mutated in vitro or in vivo, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations

in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further in vitro modification. Preferably, such mutations enhance the functional activity of the mutated gene product. Any technique for mutagenesis known in the art can be used, including but not limited to in vitro site-directed mutagenesis (25, 26, 27, 28). PCT techniques are preferred for site directed mutagenesis (29).

The identified and isolated gene can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Examples of vectors include, but are not limited to E. coli bacteriophages such as lambda derivatives, or plasmids such as pBR322 derivatives or pUC plasmid derivatives e.g., pGEX vectors, pmai-c, pFLAG, etc. The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. If the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini;

these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated. Preferably, the cloned gene is contained on a shuttle vector plasmid, which produces for expansion in a cloning cell, e.g., E. coli, and facile purification for subsequent insertion into an appropriate expression cell line, if such is desired. For example, a shuttle vector, which is a vector that can replicate in more than one type of organism, can be prepared for replication in both E. coli and Saccharomyces cerevisiae by linking sequences from an E. coli plasmid with sequences from the yeast 2 plasmid.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, cleavage [e.g., of signal

sequence]) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce a nonglycosylated core protein product. However, the transmembrane protein expressed in bacteria may not be properly folded. Expression in yeast can produce a glycosylated product. Expression in eukaryotic cells can increase the likelihood of "native" glycosylation and folding of a heterologous protein. Furthermore, different vector/host expression systems may affect processing reactions, such as proteolytic cleavages, to a different extent.

Vectors are introduced into the selected host cells for expression of the desired product by methods known in the art, e.g., transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, or a DNA vector transporter (30, 31, 32).

A recombinant product of the invention, once expressed, can be isolated and purified by standard methods. Generally it can be obtained by lysing the cell membrane with detergents, such as but not limited to, sodium dodecyl sulfate (SDS), Triton X-100, nonidet P-40 (NP-40), digoxin, sodium deoxycholate, and the like, including mixtures thereof.

Solubilization can be enhanced by sonication of the suspension. Soluble forms of the product can be obtained by collecting culture fluid, or solubilizing inclusion bodies, e.g., by treatment with detergent, and if desired sonication or other mechanical processes. The solubilized or soluble product can be isolated using various techniques, such as polyacrylamide gel electrophoresis (PAGE), isoelectric focusing, 2-dimensional gel electrophoresis, chromatography (e.g., ion exchange, affinity, immunoaffinity, and sizing column chromatography), centrifugation, differential solubility, immunoprecipitation, or by any other standard technique for the purification of proteins.

The present invention further provides for characterizing a product of the invention. For example, once a recombinant which expresses the selected gene sequence is identified, the recombinant product, which can be produced in relatively large quantities, can be analyzed. This is achieved by assays based on the physical or functional properties of the product, including radioactive labelling of the product followed by analysis by gel electrophoresis, immunoassay, etc.

The structure of a product of the invention can be analyzed by various methods known in the art. Structural analysis can be performed by identifying sequence similarity with other known proteins. The degree of similarity (or homology) can provide a basis for predicting structure and function of marker, or a domain thereof. In a specific embodiment, sequence comparisons can be performed with sequences found in GenBank, using, for example, the FASTA and FASTP programs (33).

As the description of this invention progresses, it will be apparent that many of the products of this invention can be characterized as peptides or polypeptides. Often such products

contain a relatively small number of amino acid residues, e.g. up to about 80. It may be more convenient to chemically synthesize such products rather than produce them using the recombinant techniques described above.

Polypeptide and peptides within the scope of the invention containing, for example from about 6 to 80 or more amino acid segments, may be synthesized by standard solid phase procedures with appropriate amino acids using the protection, deprotection and cleavage techniques and reagents appropriate to each specific amino acid or peptide. A combination of manual and automated (e.g., Applied Biosystem 430A) solid phase techniques can be used to synthesize the novel peptides of this invention. Although less convenient, classical methods of peptide synthesis can also be employed. For background on solid phase techniques, see citations 34 to 37.

It has now been discovered that calreticulin; the C-domain of calreticulin; segments of calreticulin containing the C-domain, for example the P&C domain; and segments of the C-domain

are useful to treat patients in need of such treatment to inhibit restenosis when such products are deposited in or about the site of vascular wall damage.

Patients in need of treatment in accordance with the invention comprise those who have undergone PTCA in which an arterial blockage has been opened by inserting a balloon tipped catheter and inflating the balloon or other interventional procedure. As has been mentioned above large numbers of these patients require one or more subsequent treatments because more plaque forms at the site of the vascular damage to again occlude the artery. The process is called restenosis.

The process of this invention is employed to inhibit, and in most cases, prevent restenosis. The result is achieved by depositing one or more of the above defined products in or about the site of the original stenosis.

A number of catheters are available for administering the active agent. One such catheter is commercially available from Advanced Catheter Systems of Santa Clara, California. Another is

the Wolinsky Catheter described in U.S. Patent 4,824,436. It is available through C.R. Bard, Inc. of Billerica, Massachusetts. This latter catheter is preferred because it is capable of depositing the active agent in the cell wall where it is immediately available at high concentration.

Although administration by a catheter is the preferred method because it can be used immediately prior to PTCA as a step in the same operative procedure, it is not the only method of administration. Parenteral administration is also applicable because it appears that the active product, or at least effective portion of it targets the area of vascular damage once it enters the blood stream. Intravenous administration is preferred although other parenteral routes such as subcutaneous or intramuscular may also be employed.

The products of the invention are amphoteric. They can exist and be utilized as free bases or as pharmaceutically acceptable metallic or acid addition salts. The pharmaceutically acceptable salts include the conventional non-toxic salts or the quaternary ammonium salts of non-toxic inorganic or organic

acids. For example, such conventional non-toxic salts include those derived from inorganic acids such as hydrochloric, hydrobromic, sulfuric, sulfamic, phosphoric, nitric and the like and the salts prepared from organic acids such as acetic, propionic, succinic, glycollic, stearic, lactic, malic, tartaric, citric, ascorbic, maleic, hydromaleic, phenylacetic, glutamic, benzoic, salicylic, sulfanilic, 2-acetoxybenzoic, fumaric, toluenesulfonic, methanesulfonic, ethane disulfonic, oxalic, isothionic, and the like.

The pharmaceutically acceptable salts of the present invention can be synthesized from the amphoteric compounds by conventional chemical methods. Generally, the salts are prepared by reacting the free base or acid with stoichiometric amounts or with an excess of the desired salt forming inorganic or organic acid or base in a suitable solvent or various mixtures of solvent. Typical bases include alkali or alkaline earth metal hydroxide e.g., sodium, potassium, lithium, calcium, or magnesium, or an organic base such as an amine, e.g.,

dibenzylethylenediamine, trimethylamine, piperidine, pyrrolidine, benzylamine and the like, or a quaternary ammonium hydroxide such as tetramethyl ammonium hydroxide and the like.

Typically the compounds of this invention are administered to a mammalian subject, e.g., a human patient, in aqueous suspensions or solutions which may contain additional reagents such as emulsifying or suspending agents.

For intramuscular, subcutaneous or intravenous use, sterile solutions or suspensions of the active ingredient are usually prepared, and the pH of the solutions should be suitable adjusted and buffered. For intravenous use, the total concentration of solutes should be controlled in order to render the preparation isotonic.

The compositions of the invention may contain the stenosis inhibiting product, i.e., any product of the invention as the only active compound, or it may contain such product as the principal active ingredient together with anticoagulants such as heparin or warfarin. The compositions may also contain

thrombolytic agents such as plasminogen activators or streptokinase to inhibit platelet aggregation. All such compositions are within the scope of the invention.

The compositions of the invention may contain varying amounts of active compounds. The important criterion is that the compositions be prepared to deliver an amount of the selected compounds which is effective to elicit an anti-restenosis response. The appropriate amount may be readily determined by the skilled artisan and it may vary depending upon the age, weight and other factors pertaining to the individual patient.

Certain of the active products of this invention are novel, others are known. Calreticulin, itself and several methods for its preparation have been known since 1974. The preparation of the various domains has been described by Bakish and Michalak (3). The novel active segments of the C-domain can be prepared by conventional chemical synthesis or recombinant techniques as described herein.

The active segments are new and generally comprise segments of the C-domain of calreticulin containing from about 6 to about 80 amino acid residues. Such novel compounds may be further characterized as having the same, functional anti-restenosis activity as the C-domain of the reticulin. While we do not wish to be bound by theory, it appears that there may be a receptor in damaged vascular tissue or platelets or other circulating mononuclear cells involved in plaque formation which react with an exposed sequence in the C-domain of calreticulin. This sequence is exposed and reactive in calreticulin or any segment of calreticulin which contains the C-domain. The novel products of this invention are useful because they contain amino acid sequences having the same or similar reactivity as the exposed sequence of the C-domain. Some of novel products are more active than the C-domain, some are less active.

As has been explained above, and as will be readily apparent to the skilled artisan, a novel C-domain segment of this invention may differ slightly from the native segment in the C-domain and still have similar activity. It may contain more or fewer amino acid residues, or one or more of the amino acid

residues may be replaced with another residue. Generally, however, they will have substantially the same number of amino acid residues and a similar tertiary structure so that the correct sequence is exposed and reactive. The compounds may be defined as polypeptides which are substitution, addition or deletion analogs of the C-domain.

The invention contemplates not only compositions and the use of compositions of known products to control restenosis. It also encompasses the novel compounds aforesaid, therapeutically effective compositions containing such novel compound or compounds, transformed cell lines useful for the expression of the novel compounds, DNA molecules encoding such novel compounds and plasmids or other vectors incorporating such molecules.

The process of the invention will be understood from consideration of the following study which was conducted to establish the efficacy of the calreticulin and the C-domain thereof to inhibit restenosis.

Fifty nine Sprague Dawley rats were studied in total; 10, rats for Study 1, 24 rats for Study 2, and 25 rats for Study 3. Each rat had introduction of a 1.5 mm diameter, 20 mm length angioplasty balloon (USCI) retrograde, via femoral arteriotomy, to the iliac arterial ostium at the iliac bifurcation under general pentobarbitol anesthetic (6.5 mg per 100 g weight by i.m injection, Somnotrol, MTC Pharmaceuticals, Cambridge, Ontario, Canada). Infusions (1.0 m.l. total volume) were given immediately prior to balloon inflation through the distal central lumen of the angioplasty balloon catheter upstream of the site for balloon mediated damage. After infusion of calreticulin and each of the other proteins and protein fragments tested, the balloon was positioned just below the iliac bifurcation across the left iliac and femoral artery branches and inflated to 8 bars pressure for 1.0 minute.

The infusions were give for each study protocol as follows:
Study 1: To assess the capacity of calreticulin to interfere with atherosclerotic plaque development after endothelial injury, 0.01 mg of calreticulin (5 rats) or saline control (5 rats) was

infused by intra-arterial injection through the distal lumen of the angioplasty balloon catheter upstream from the site of subsequent balloon mediated damage. Table 1.

Study 2: In the second series of experiments, 0.0001, 0.001, 0.01 and 0.1 mg of calreticulin was given to each of two rats (8 rats total), 0.0001, 0.001, 0.01, and 0.1 mg of calsequestrin a similar calcium binding protein was given to each of 2 rats (8 rats total), or saline (8 rats), by local infusion into the iliac and femoral artery through the distal tip of the angioplasty catheter. Table 2.

Study 3: In order to determine the active site in the calreticulin molecule for inhibition of plaque growth, 0.1 mg of the C (4 rats), P (4 rats), P (4 rats) and N (4 rats) calreticulin protein domains as well as both human (4 rats) and rabbit (4 rats) calreticulin and saline (5 rats) were similarly infused upstream into the ilio-femoral arterial branch prior to balloon injury.

After infusion and angioplasty, the femoral artery was tied off with 3-0 silk in Study 1 and the arteriotomy site was sealed with local application of n-butyl cyanoacrylate monomer

(Nexaband, Veterinary Products Laboratories, Phoenix, Artzone) for Studies 2 and 3. Each rat was maintained on a normal rat diet and was followed up for 4 weeks post surgery. The rats were not given heparin nor other anti-platelet agents during this study. At follow up, the rats were sacrificed with 2.0ml euthanyl per kg and the aorta was harvested for histological examination. After sacrifice, the distal abdominal aorta and the bilateral iliac and femoral arteries were harvested, divided into the left and right ilio-femoral arterial branches, the bifurcation, and the distal abdominal aorta, and fixed in neutral buffered formalin. This protocol was reviewed and approved by the University of Alberta Health Sciences Laboratory Animal Services laboratory animal welfare committee. All animal studies conformed to Canadian guidelines for laboratory animal experimentation.

Calreticulin - Source and Purification:

Calreticulin was purified by the ammonium sulfate precipitation procedures as described earlier (38, 39). Canine cardiac calsequestrin was prepared from whole tissue homogenates

in the presence of protease inhibitors by the published procedure (40) (1987). Recombinant full-length calreticulin was expressed in E. coli as GST fusion proteins and purified (3, 40).

The domains of calreticulin and recombinant GST were expressed in E. coli and purified (3). In this study, we have expressed, as GST-fusion proteins, three domains of calreticulin: N-domain and the C-domain. Goat anti-rabbit calreticulin antibody was prepared as described by Milner et al. (38). Protein was determined by the method of Lowry et al. (41) or Bradford (42).

Histology and Morphometric Analysis:

Each specimen was fixed in 10% sodium phosphate buffered formalin, processed, impregnated and embedded in paraffin and cut into 5 um sections by microtome in accordance with standard procedures. The iliac and femoral arterial branches were removed and divided into three sections (proximal, mid, and distal) and stained with hematoxylin and eosin for light microscopic and morphometric examination. For morphometric analysis, the section with the largest detectable area of cellular proliferation and

atherosclerotic plaque (out of the 3 sections taken from each rat) detectable by light microscopy was outlined using a Nikon Optiphot-Labophot drawing device microscope attachment attached to a Nikon Model Labophot - 2 light microscope (Nikon, Nippon Logaku K.K., Tokyo, Japan). Each drawing was standardized to a 1mm marker measured through the 10X objective used for the morphometric analysis drawings. Atherosclerotic plaque area and thickness were measured using a Jandel Scientific Sigma Scan program and Summagraphics digitizing Summa sketch pad coupled to a Mac IICx computer. All histologic sections were independantly assessed by a blinded observer, vetrinary pathologist, for pathologic changes.

Statistics:

Correlations between the measured plaque area as well as observed stenoses as documented by angiography were assessed both by ANOVA, Fisher PSLD, and Student's t test. The incidence of atherosclerotic lesions detected on histological examination was assessed by chi square analysis.

Results:**Reduced Intimal Hyperplasia after Local Infusion of Calreticulin at Sites of Balloon Induced Endothelial Injury:**

Study 1: There was a significant reduction in plaque development after calreticulin infusion. 5/5 Saline fused control rats developed fibrotic intimal proliferation with hemosiderin laden macrophage, infiltration, and collateral artery development. The fifth specimen had evidence of arterial injury with aneurysmal dilatation, macrophage infiltration and collateral development but minimal intimal hyperplasia. 2/5 of the calreticulin treated rats had plaque development ($P < 0.001$). The overall plaque area in the control rats was $0.004 \pm 0.001 \text{ mm}^2$ whereas in the calreticulin infused rats the mean plaque area was $0.001 \pm 0.001 \text{ mm}^2$ ($p = \text{NS}$). Similarly there was a trend toward decreased plaque thickness, the intimal thickness was $0.044 \pm 0.027 \text{ mm}$ after calreticulin infusion and $0.108 \pm 0.063 \pm 0.028 \text{ mm}$ after saline infusion ($p = \text{NS}$). (Table 1). There was no evidence for thrombosis at any of the sites previously infused with calreticulin or saline. The contralateral arteries that did not

have surgical intervention were normal in all but one of the ten rats in Study 1. The areas of injury induced plaque development were characterized by changes in cellular proliferation and fibrous plaque development but there was no apparent fatty or complex plaque development.

Study 2: The second series of experiments was performed to confirm whether there was a true inhibition of plaque growth after calreticulin infusion. There was again a decrease in the incidence of detectable plaque in rats with calreticulin infusion; 8/8 of the calsequestrin and saline infused rat arteries had microscopic evidence of plaque development at 4 weeks follow up whereas 3/8 rats that had calreticulin infusions had no detectable plaque in any of the sections examined. In the calreticulin infused sections there was overall, a decrease in the extend of intimal cellular hyperplasia and the size of developing plaque, as assessed by morphometric analysis of plaque area and thickness, on comparison with saline infusion (p 0.02 by ANOVA).

Study 3: The experiments performed in Study 3 were used to assess the efficacy of the various protein domains on plaque growth after arterial injury. As a control, calreticulin, both human and rabbit, was again infused as was saline in order to confirm prior to results. There was again a decrease in the incidence of observed plaque development in histological sections taken from rats that received infusions of either human or rabbit calreticulin on comparison with saline infusion. 5/5 Saline infused rats whereas 2/4 of the human calreticulin infused and 3/4 of the rabbit calreticulin infused rat arterial sections had detectable plaque. A significant decrease in both plaque area and plaque thickness was detected after infusion of calreticulin, both human and rabbit (p 0.0066 for plaque thickness, p 0.0004 for plaque area by ANOVA) on comparison with saline infusion.

Dose Related Inhibition of Intimal Hyperplasia after Calreticulin Infusion:

There was a significant decrease in calreticulin treated rat femoral arteries in the second study series with a clearly dose related response. See Table 2. In addition the effect of

calreticulin was titrated out at the lower dose infusions (Table 2). Histological specimens had graded decreases in the measured plaque area at 0.01 to 0.1 mg doses of calreticulin, but minimal decrease to no decrease in plaque growth after 0.001 and 0.0001 mg dose infusion, respectively. The fact that calreticulin effects were titrated out at the lower doses indicated that the observed anti-proliferative effects were dose dependant.

Calsequestrin, A Ca^{2+} binding protein similar to calreticulin has minimal effect on plaque development:

Calsequestrin is a Ca^{2+} binding protein of the muscle sarcoplasmic reticulum membrane (43). Calsequestrin and calreticulin share a number of physicochemical properties including their high capacity Ca^{2+} binding sites and their intracellular localization (44). In contrast to calsequestrin, calreticulin is a KDEL protein and it was shown to interact with a variety of different cellular proteins (45). Calsequestrin was infused into the femoral artery to assess the capacity of a Ca^{2+} binding protein similar to calreticulin to prevent plaque growth after balloon mediated injury. Suprisingly, the inhibitory effect of calreticulin was not reproduced by a related Ca^{2+}

binding protein, calsequestrin indicating that the anti-, proliferative effect was not produced by Ca^{2+} binding associated action. There was no comparable decrease in plaque growth in any of the arteries where calsequestrin was infused prior to balloon injury. The mean plaque area was $0.042 \pm 0.025 \text{ mm}^2$ after saline infusion, $0.034 \pm 0.069 \text{ mm}^2$ after calsequestrin infusion, and $0.014 \pm 0.016 \text{ mm}^2$ after calreticulin infusion (p 0.03 by ANOVA, 95% confidence for comparison of calreticulin and calsequestrin at doses greater than 0.001 mg or calreticulin and saline but no significant difference for calsequestrin and saline by Fisher test. There was no decrease in plaque formation at either high or low doses of calsequestrin infusion or after saline infusion. At the highest dose of calsequestrin infused some decrease in plaque development at 4 weeks was observed. The plaque that developed in the calsequestrin treated rats was again intimal hyperplasia for the most part. Calsequestrin is a very efficient Ca^{2+} binding protein and at the doses given should have had comparable Ca^{2+} binding properties to the calreticulin infused.

Analysis of the Domains of Calreticulin for Anti-Proliferative Activity.

After infusion of the C, N and P domains of calreticulin a decrease in subsequent plaque development was observed only after C domain infusion. 0/4 of the specimens taken from rats that had C domain infusion had evidence of intimal hyperplasia, while 4/4 of either P or N domain infused rats had evidence for plaque growth on microscopic examination (Table 3). Significant differences were also calculated for the plaque area after C domain infusion on comparison with the saline and P or N, 95% confidence by Fisher PSDL domain infusions but no significant difference was detectable on comparison with CRT infusion whether rabbit or human or on comparison of infusion of the C domain with rabbit or human calreticulin (p 0.0001 for plaque area, p 0.01 for plaque thickness by ANOVA). The decrease in plaque was greater after C domain than after either human or rabbit calreticulin infusion, but comparison of the plaque areas after C domain infusion with CRT (human and rabbit) was not significant (Fisher PSDL).

Similar results are obtained with studies on the P & C domain (inhibition of restenosis), the N & P domain (no inhibition) and with a number of segments from the C-domain

containing at least 6 amino acid residues. Best results are obtained with segments containing at least 6 amino acid residues the amino terminal of which is amino acid residue 331 to 391 of 346 to 401 of the C-domain. Of the novel materials tested, the best results were obtained with KEEEEEEKKRKEEEAEDEEDKDDKEDEDED-EEDKDEEEEEE.

The presently preferred products are polypeptides from the carboxy end of the C-domain containing from about 10 to about 45 amino acid residues, principally acidic amino acid residues because they are generally easier to prepare, appear to be very active and do not interfere with the other activities of calreticulin.

Calreticulin, the C-domain of calreticulin and the P & C domain of calreticulin were produced with conventional recombinant procedures (3) utilizing transformed E. coli which have been deposited at the ATCC under the numbers 69823, 69822 and 69821 respectively. Other active compounds of the invention are similarly prepared.

• The following citations all of which are incorporated by reference are cited in this disclosure.

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WHAT IS CLAIMED IS:

1. A method of treating a patient to inhibit restenosis which comprises administering to such patient in an amount which is effective to inhibit restenosis a compound selected from the group consisting of calreticulin; the C-domain of calreticulin; a C-domain containing segment of calreticulin; and a polypeptide which contains from about 6 to 100 amino acid residues and is an addition, substitution or deletion analog of the C-domain of calreticulin having the same functional activity.

2. A method according to claim 1 wherein the compound is administered utilizing a catheter.

3. A method according to claim 1 wherein the compound is administered parenterally.

4. A method according to claim 3 wherein the compound is administered intravenously.

5. KEEEEKKRKEEEEAEDEEDKDDKEDEDEDEEDKDDEEEEE.

1 \ 1

10 20 30 40 50
 EPVVYFKEQF LDGDGWTERW IESKHKSDFG KFVLSSGKFY GDQEKDKGLQ

N-domain

60 70 80 90 100
 TSQDARFYAL SARFEPFSNK GQPLVVQFTV KHEQNIDCGG GYVKLFPPAGL

N-domain

110 120 130 140 150
 DQKDMHGDSE YNIMFGPDIC GPGTKKVHVI FNYKGKNVLI NKDIRCKDDE

N-domain

160 170 180 190 200
 FTHLYTLIVR PDNTYEVKID NSQVESGSLE DDWDFIPPKK IKDPDASKPE

210 220 230 240 250
 DWDERAKIDD PTDSKPEDWD KPEHIPDPDA KKPEDWDEEM DGEWEPPVIO

P-domain

260 270 280 290 300
 NPEYKGEWKP ROIDNPDYKG TWIHPEIDNP EYSPDANIYA YDSFAVLGLD

P-domain

310 320 * 330 340 350
 LWQVKSGTIF DNFLITNDEA YAEFFGNETW GVTKTAEKQM KDKQDEEQRL

C-domain

360 370 380 390 401
 KEEEEKKRK EEEEEEDDEE DKDDKEDEDE DEEDKDEEEE EAAAGQAKDEL

C-domain**FIG. 1**

INTERNATIONAL SEARCH REPORT

 International Application No
 PCT/IB 96/00471

 A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C07K14/47 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

 Minimum documentation searched (classification system followed by classification symbols)
 IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 266, no. 32, 15 November 1991, MD US, pages 21458-21465, XP002015478 BAKSH S. ET AL.: "Expression of Calreticulin in Escherichia coli and Identification of Its Ca ²⁺ Binding Domains" cited in the application see the whole document --- -/--	1-5

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

10 October 1996

Date of mailing of the international search report

18. 10. 96

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Moreau, J

INTERNATIONAL SEARCH REPORT

Internat. Application No.

PCT/IB 96/00471

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY 270 (36). 1995. 21404-21410, XP002015479 PATTON W F ET AL: "Components of the protein synthesis and folding machinery are induced in vascular smooth muscle cells by hypertrophic and hyperplastic agents: Identification by comparative protein phenotyping and microsequencing." see the whole document ---	1-4
P,A	US 5 426 097 A (STERN D.M. ET AL.) 20 June 1995 see the whole document -----	1-4

INTERNATIONAL SEARCH REPORT

International application No.

PC./IB 96/00471

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1 - 4 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Application No

PCT, IB 96/00471

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-5426097	20-06-95	NONE	